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New reports of *Inocybe* from pine forests in Pakistan

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Abstract — During investigations of ectomycorrhizal fungi associated with pine trees in Pakistan, *Inocybe amicta* and *I. mimica* were collected underneath *Pinus wallichiana*. Both morphological and nrDNA ITS molecular evidence support the identity of these species. They represent new records from pristine forested areas of Pakistan, and from Asia. *Pinus wallichiana* is recorded as a new host for both species. Twenty-six *Inocybe* taxa have now been reported from Pakistan.

Key words — *Agaricales*, *Basidiomycota*, *Inocybaceae*, Shangla

Introduction

The family *Inocybaceae* Jülich, containing the genera *Inocybe* (Fr.) Fr., *Auritella* Matheny & Bougher, and *Tubariomyces* Esteve-Rav. & Matheny, is a highly diverse monophyletic group of ectomycorrhizal fungi that comprises between 500 and 700 species worldwide (Matheny et al. 2009, Alvarado et al. 2010). Between 70% and 80% of species in the family have been described from the north temperate zone in association primarily with the ectomycorrhizal (ECM) plant families *Pinaceae*, *Fagaceae*, and *Salicaceae*. However, most studies on fungal ecology and biodiversity have focused on taxa outside the tropics, a region where ECM fungal taxa deserve more attention (Alexander & Selosse 2009, Smith et al. 2011).

Inocybe is a large genus of gilled fungi that includes many fairly small and inconspicuous brown species (Kokkonen & Vauras 2012). Matheny (2009) identified seven clades within *Inocybaceae:* /inocybe, /nothocybe, /pseudosperma, /mallocybe, /inosperma, /mallocybella, and /auritella.

Previously, *I. adaequata* (Britzelm.) Sacc., *I. argillacea* (Pers.) Singer, *I. asterospora* Quél., *I. bongardii* (Weinm.) Quél., *I. dulcamara* (Pers.) P. Kumm.,

I. fastigiata (Schaeff.) Quél., *I. fibrosa* (Sowerby) Gillet, *I. flocculosa* Sacc., *I. fuscidula* Velen., *I. geophylla* (Bull.) P. Kumm., *I. glabripes* Ricken, *I. hirtella* Bres., *I. microspora* J.E. Lange, *I. napipes* J.E. Lange, *I. nitidiuscula* (Britzelm.) Lapl., *I. oblectabilis* (Britzelm.) Sacc., *I. patouillardii* Bres., *I. posterula* (Britzelm.) Sacc., *I. praetervisa* Quél., *I. pyriodora* (Pers.) P. Kumm., *I. splendens* R. Heim, *I. splendentoides* Bon, *I.` squamata* J.E. Lange, and *I. vaccina* Kühner have been reported from Pakistan (Ahmad et al. 1997, Sultana et al. 2011, Ilyas et al. 2013). During our present exploration of ectomycorrhizal fungi from pine forests of the western Himalayas, *I. amicta* and *I. mimica* were found associated with *Pinus wallichiana* A.B. Jacks. Both morphological and molecular evidence were used to confirm the identity of these species. These records raised the number of *Inocybe* spp. reported from Pakistan to twenty-six.

Materials & methods

Morphological evaluation

Basidiomata were collected, photographed, vouchered in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH), dried, and characterized morphologically. Sections of specimens were mounted in 5% KOH for microscopic observations under a biological microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Phloxine was used to increase contrast of the structures. Measurements of anatomical features (basidiospores, basidia, cystidia, pileus, and stipe hyphae) are presented from at least 25 measurements made with an ocular micrometer and 100 oil-immersion objective, and include x = arithmetic mean of spore length and spore width for all spores measured, Q = spore length divided by spore width. Line drawings were made with a camera lucida. Color designations are from Munsell (1975).

DNA extraction, PCR amplification, DNA sequencing

Genomic DNA was extracted from a small piece of pileus by a modified CTAB method (Bruns 1995). Amplification of internal transcribed spacers (ITS1 & ITS2) and the 5.8S region of the nuclear ribosomal RNA gene were targeted using the primer pairs ITS1F and ITS4 (White et al. 1990, Gardes & Bruns 1993) using the Extract-N-Amp plant DNA extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). PCR was carried out under the following cycling parameters: initial denaturation (94°C for 1 min), 35 cycles (94°C for 1 min, 53°C for 1 min, and 72°C for 1 min), and final extension (72° C for 8 min). Amplified PCR products were sent for purification and bidirectional sequencing to Macrogen (Republic of Korea).

Sequence alignment and phylogenetic analysis

Sequencing of the nrITS region of our four Pakistani *Inocybe* collections yielded fragments of 640–670 base pairs. GenBank sequences for alignment and phylogenetic analysis were selected based on the top 100 BLAST search results for our four new sequences.

Fig. 1. Basidiomata: A. ***Inocybe amicta*** (MSM0010); B. ***Inocybe mimica*** (MSM0011). Scale bars: A = 7 mm; B = 15 mm.

Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). Following Dentinger et al. (2011) for complete ITS sequences, all sequences were trimmed with the conserved motifs 5′-(…GAT) CATTA– and –GACCT (CAAA…)-3′ and the alignment portion between them was included in analysis. *Simocybe serrulata* (Murrill) Singer was used as outgroup based on results reported by Larsson et al. (2009).

One sequence of *Inocybe amicta*, three sequences of *I. mimica,* and other related sequences retrieved from the GenBank were aligned by Muscle using the default setting in Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2011). A phylogenetic tree was constructed with the Maximum Likelihood (ML) algorithm using a Jukes & Cantor (1969) model of nrITS sequences and nearest-neighbor-interchange (NNI) as ML heuristic search method using MEGA5 software (Tamura et al. 2011). The topology was assessed by 1000 bootstrap replicates. Corresponding bootstrap values >50 % are cited on the trees (Figs 5, 6).

Sequences of *I. amicta* and *I. mimica* generated for this study were submitted to GenBank. Accession numbers for the taxa retrieved from GenBank used in the phylogenetic analysis are cited in the phylogenetic trees. Percent identities and DNA divergences were calculated by DNAStar.

Taxonomy

Inocybe amicta Kokkonen & Vauras, Mycol. Progr. 11: 323 (2012). Figs 1A, 2, 4A,B

Pileus 25–35 mm diam., conical or conic-convex, with an obtuse umbo; margin reflexed, rimulose, surface dull, smooth at the disc, torn or cracked towards the margin; brown, dark brown or reddish brown (2.5YR1/4) at the center, fading to grayish brown or strong brown (5YR4/6) towards the margin. Lamellae adnexed, moderately crowded, with one tier of lamellulae, pale brown (5YR4/6), edge even, concolorous. Stipe 50–84 mm long, 6 mm diam. at the apex, 10 mm diam. at the base, which is rounded subbulbous; central, solid; surface not pruinose, white felted or floccose, striate; dark brown (5YR4/6).

Basidiospores 8–9.6 × 5.6–7 µm [x = 8.9 × 6.4 µm, Q = 1.20–1.56], weakly to strongly nodulose, nodules vary in size, pale yellowish brown in KOH, inamyloid. Basidia 22.8–32 × 6.1–12.5 µm, clavate, four-spored, thin-walled, hyaline in KOH; sterigmata 3.3–6.3 µm. Pleurocystidia 49–68 × 15–21 µm, metuloid, lageniform with short neck or utriform rarely fusiform, apex encrusted with crystals, tapered at the base. Cheilocystidia 47–66 × 19–28 µm, similar to pleurocystidia. Pileipellis a cutis, hyphae cylindrical, thin-walled, hyaline in KOH. Caulocystidia 24–59 × 7–18 µm, present at the stipe apex, cylindrical or subfusiform, hyaline in KOH, hyphoid cystidia common, often with septa, wall thick at base or with protuberances in center. Clamp connections present.

Material examined: **PAKISTAN**, **Khyber Pakhtoonkhaw**, Shangla, under *Pinus wallichiana,* 2 September 2013, coll. M. Saba & A.N. Khalid MSM0010 (LAH; GenBank KJ686344).

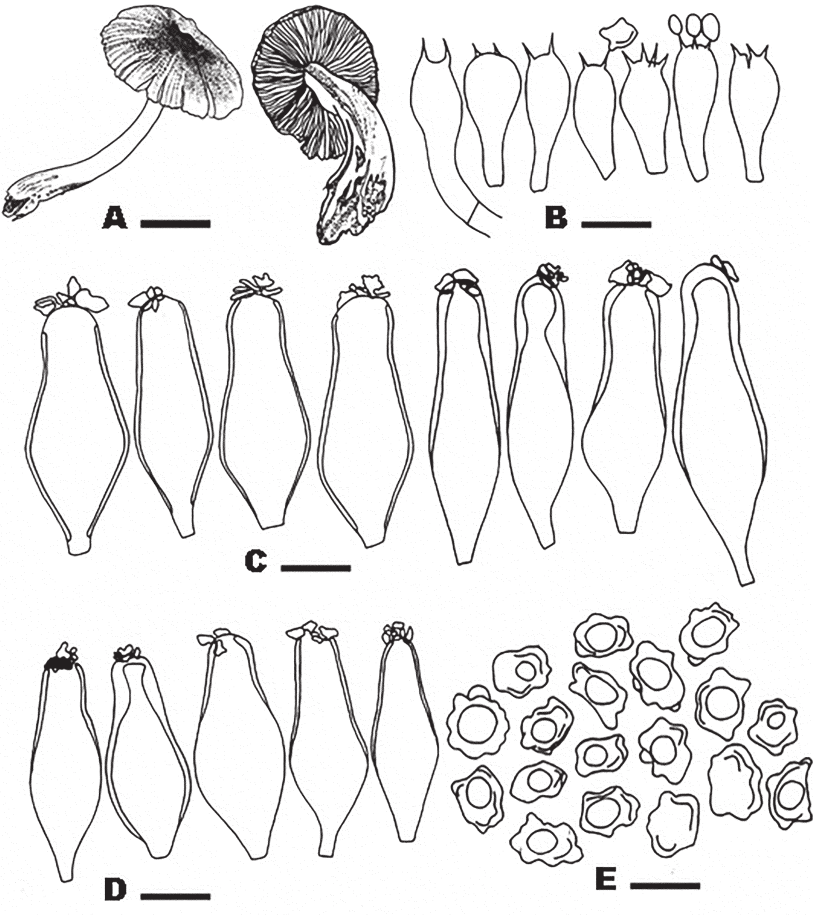

Fig. 2. *Inocybe amicta* (MSM0010):
A. Basidiomata; B. Basidia; C. Pleurocystidia; D. Cheilocystidia; E. Basidiospores.
Scale bars: A = 7 mm; B = 12 µm; C = 15 µm; D = 20 µm; E = 8 µm.

Inocybe mimica Massee, Ann. Bot. 18: 492 (1904). Figs 1B, 3, 4C,D

Pileus 31–35 mm diam., conical, obtusely umbonate, margin reflexed, bent downward; surface radially fibrillose, striate, shiny, with a few scattered scales, cracked towards the center; pale greenish/brownish yellow (7.5Y9/4). Lamellae sinuate or adnexed, close, pale greenish yellow (7.5Y9/4), edge wavy, white fimbriate. Stipe 55–72 mm long, 6–8 mm diam. at the apex, 9–14 mm diam. at the base, central, equal, with a rounded subbulbous base, solid;

surface longitudinally striate, fibrillose, off-white in the upper part and with pale greenish yellow in the lower part.

Basidiospores (9.2–)10–14.1 × 6.6–8 µm [x = 12.5 × 9.8 µm, Q = 1.4–1.96], mostly ellipsoid, apiculus present, smooth, yellowish brown or pale brown in KOH, inamyloid. Basidia 9.9–55.4 × 9.6–17.2 µm, clavate, four-spored, thin-walled, hyaline in KOH; sterigmata up to 5.1 µm long. Pleurocystidia absent. Cheilocystidia 33.2–74.5 × 10.8–23.6 µm, narrowly clavate or cylindrical, smooth, hyaline in KOH. Pileipellis a cutis, hyphae cylindrical, 6.6–21 µm, thin-walled, hyaline in KOH, some with encrusting pigment. Caulocystidia 33.7–65 × 9.9–15.5 µm, cylindrical or clavate, hyaline in KOH, thin-walled. Clamp connections present.

Material examined: **PAKISTAN**, **Khyber Pakhtoonkhaw**, Shangla, under *Pinus wallichiana,* 2 September 2013, coll. M. Saba & A.N. Khalid MSM0011 (LAH; GenBank KJ546158); 2 September 2013, I. Ahmad & A.N. Khalid P-48 (GenBank 726737), P-69 (KJ700456).

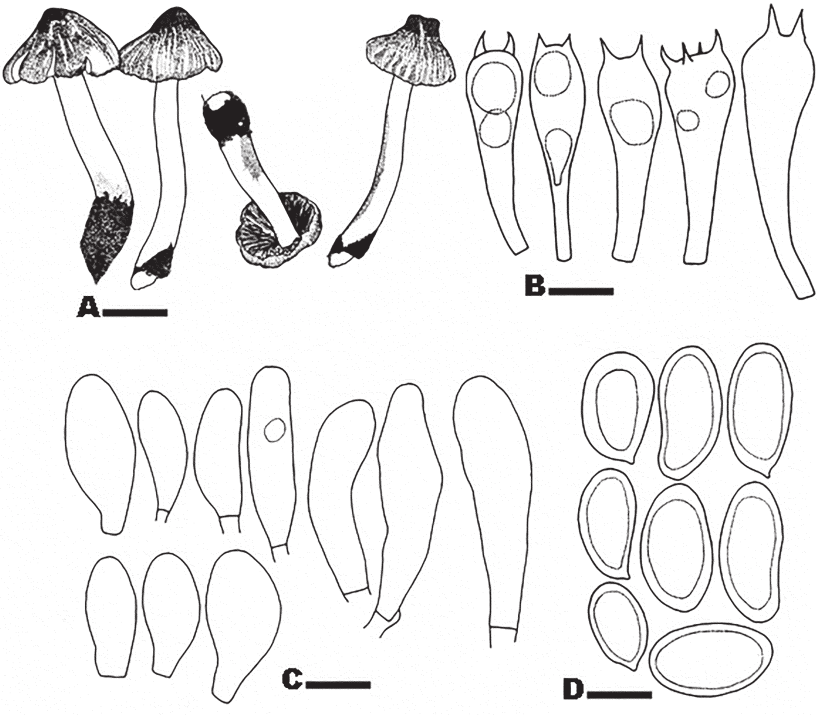

Fig. 3. *Inocybe mimica* (MSM0011):
A. Basidiomata; B. Basidia; C. Cheilocystidia; D. Basidiospores.
Scale bars: A = 15 mm; B = 10 µm; C = 18 µm; D = 6 µm.

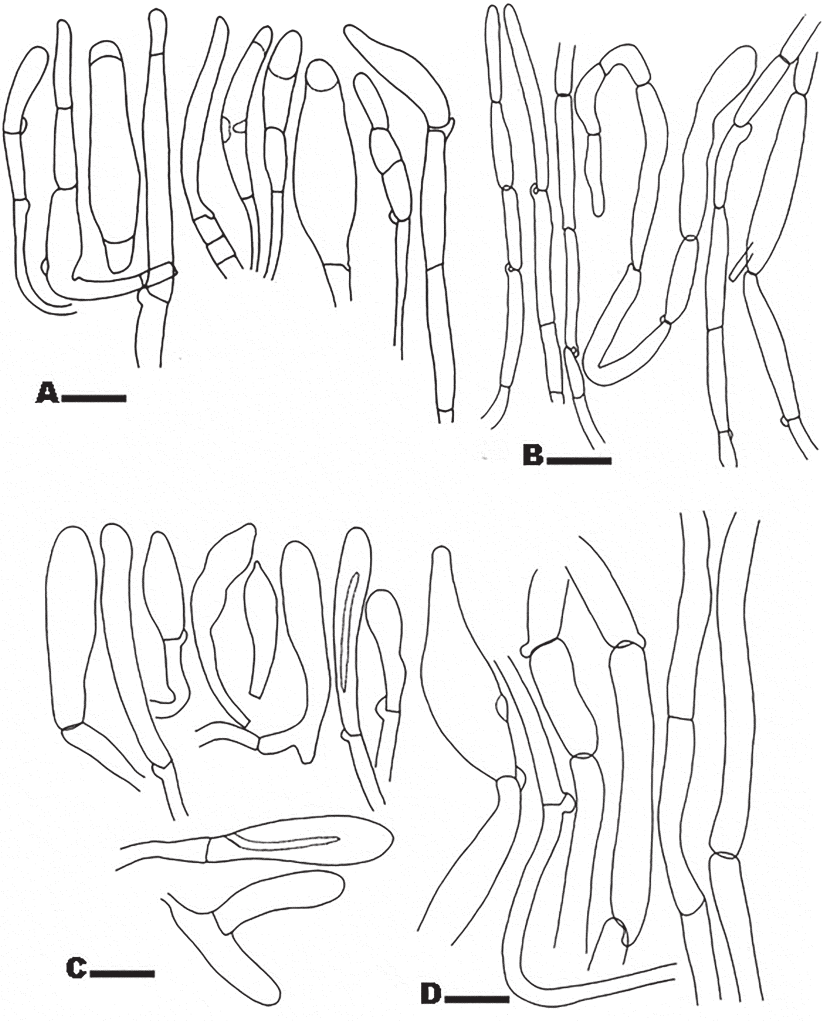

Fig. 4. ***Inocybe amicta*** (MSM0010): A. Caulocystidia; B. Pileipellis.
Inocybe mimica (MSM0011): C. Caulocystidia; D. Pileipellis.
Scale bars: A = 20 µm; B–D = 15 µm.

Results

After removing and editing ambiguous positions in the alignment, a total of 740 characters was subjected to phylogenetic analysis of which 377 were conserved, 335 were variable, 230 were parsimony informative, and 100 were singletons.

The ITS sequence of our Pakistani collection MSM0010 grouped in a clade with four GenBank sequences of *Inocybe amicta* with 100% support (Fig. 5); and the ITS sequences of our Pakistani collections MSM0011, P-48, and P-69 grouped in a clade with two GenBank sequences of *Inocybe mimica* with 98% support (Fig. 6).

Discussion

In general, *Inocybe* spp. show a high sequence divergence in the ITS region. Closely related species often deviate in several substitutions and insertion/deletion events and are therefore easy to identify using simple sequence comparison (Altschul et al. 1997).

Initial BLAST analysis of ITS sequences of our Pakistani collection MSM0011 showed 98% identity with *Inocybe amicta* sequences JF908263 from Italy and JN580861 from Finland. Our phylogenetic reconstruction based on ITS sequences shows authentic *I. nitidiuscula* well separated from *I. amicta*. Morphologically, MSM0011 has the nodulose spores and white felted or floccose stipe described for *I. amicta* (Kokkonen & Vaurus 2011), in contrast to the smooth basidiospores and whitish pruinose upper third of stipe characterizing *I. nitidiuscula* (Obase et al. 2006). We therefore conclude that HQ604600 is a sequence from a misdetermined specimen of *I. amicta*.

Inocybe amicta has been described from three localities in Finland from southern and northern boreal zones on richer but mainly sandy soils with *Betula*, *Picea abies*, and *Pinus sylvestri*s (Kokkonen & Vauras 2012). The species is grouped in clade /inocybe, which is characterized by a distinct apiculus on the spores and presence of pleurocystidia. Species with nodulose spores probably evolved independently on numerous occasions (Matheny 2005). The /inocybe clade includes species that are distributed primarily in temperate areas. This is undoubtedly the most evolutionarily diverse clade and contains approximately 85% of the species in *Inocybaceae* (Matheny 2009).

To our knowledge, this is the first report of *I. amicta* from Asia and the first record of its association with *Pinus wallichiana.* We also believe this is the first report of this species since it was first described from Finland in 2012 (Kokkonen & Vaurus 2012). This study demonstrates *I. amicta* has a wide distribution outside European countries. It is closely related to *I. silvae-herbaceae* (Fig. 5), which is distinguished by the finely fibrillose to delicately floccose stipe turning pale brown (often with a reddish tinge), and the presence of multiform caulocystidia. The ITS sequences of these two species differ only by 14 bases and 4 insertions/deletions (each 1 base in length) (Kokkonen & Vauras 2012).

Initial BLAST analysis of ITS sequences of our three Pakistani collections MSM0010, P48, and P69 revealed 99% maximum identity with *Inocybe mimica*

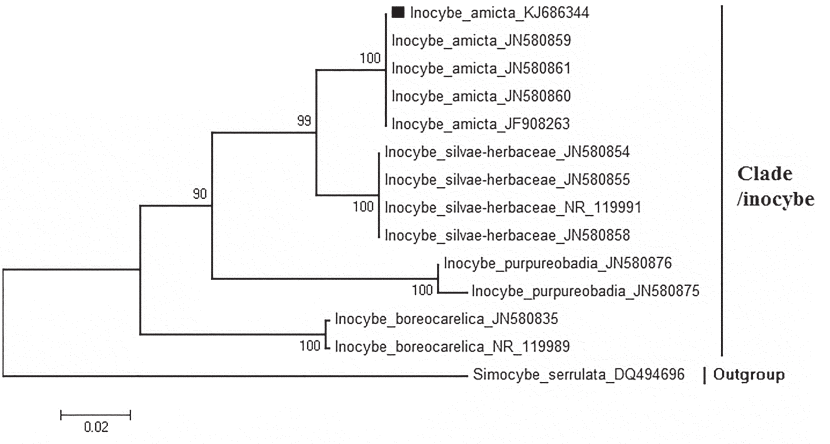

Fig. 5. Phylogenetic relationship of *Inocybe amicta* with other related *Inocybe* spp. based on Maximum Likelihood inferred from nrITS sequences.

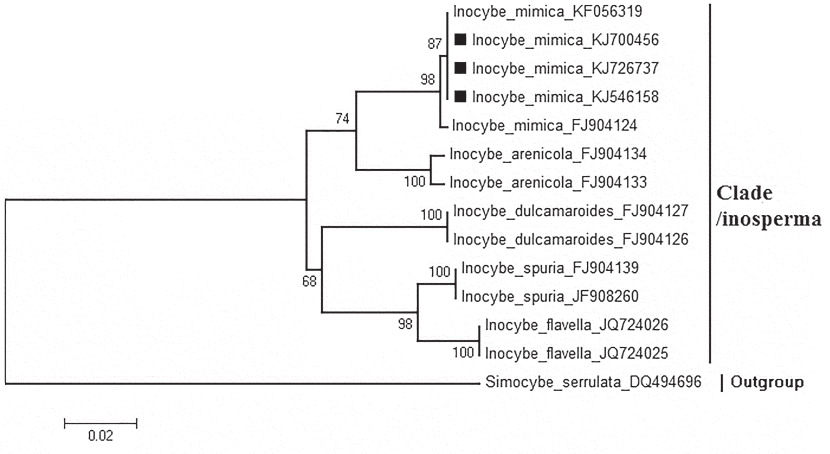

Fig. 6. Phylogenetic relationship of *Inocybe mimica* with other related *Inocybe* spp. based on Maximum Likelihood method inferred from nrITS sequences.

sequences KF056319 from India and FJ904124 from Finland, both from coniferous forests.

Inocybe mimica was described by Massee (1904) from Yorkshire, UK. Larsson et al. (2009) reported this species under *Betula*, *Picea*, and *Pinus* trees

on calcareous soils from Northwest Europe (Sweden). Our collections represent the first report of *I. mimica* from Asia and the first record of its association with *Pinus wallichiana*. According to Larsson et al. (2009), *I. mimica* is a very rare and poorly known species that is closely related to *I. arenicola* (Fig. 6). Both are characterized by rather large spores and a preference for calcareous soils, but other morphological and ecological traits are highly variable. Larsson et al. (2009) placed *I. mimica* in clade /inosperma. Molecular analysis confirms that most species in this clade bear phaseoliform spores for the most part and/or have reddening context. A rimose pileus appears to be symplesiomorphic for the clade as some species of *I.* sect. *Cervicolores*, which are derived within the group, bear a squamulose pileus (Matheny 2009).

This study raises the total number of known taxa of *Inocybe* to twenty-six in Pakistan.

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